

Characterization and isolation of a rotifer-deterrent compound in the red tide dinoflagellate *Karenia brevis*.

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Abstract

Spontaneous phytoplankton blooms cause extensive damage to aquatic ecosystems all over the world. Red Tide, a type of bloom, causes extensive damage in the Gulf of Mexico every year. Red tide is caused by *Karenia brevis*, a type of phytoplankton known to be especially hazardous because of the harmful toxins it secretes into its environment. Although this phytoplankton species, like many others, can cause extensive environmental damage, little is known about its eco-system dynamics. Studying the relationships between phytoplankton and their consumers will help to better understand the mechanisms of bloom formation. For instance, previous research has demonstrated that *Karenia brevis* deters feeding for a potential grazer, the rotifer *Brachionus plicatilis*. Further research showed that a cellular component may be responsible for defending *K. brevis* against grazers. Using bioassay- guided fractionation techniques, we have attempted to isolate and characterize the chemical responsible for this deterrence. Liquid partition yielded a deterrent, lipophilic fraction . Size- exclusion chromatography also generated an active fraction, by separating compounds by molecular size. By characterizing this unknown compound, we hope to learn more about the interactions between phytoplankton and their consumers.

Introduction

As microscopic primary producers, phytoplankton are integral members of many aquatic ecosystems. Moreover, phytoplankton can be important biomarkers for environmental changes, and they are incredible oxygen producers. However, they are probably most well known for their destructive role in phytoplankton blooms. Blooms, consisting of rapidly increasing concentrations of phytoplankton, have the potential to greatly disturb their environment.

The phytoplankton bloom, red tide, occurs frequently in the Gulf of Mexico, where it costs the US fishing industry about 18 million dollars each year (Anderson et al., 2000). The destruction is partly due to toxins, called brevetoxins, produced by the phytoplankton species, *Karenia brevis*. Besides massive fish kills, these toxins also cause neurological damage to marine mammals and health problems for humans (Sayer, Qing et al. 2005). Humans are exposed to brevetoxins when they consume brevetoxin-contaminated shellfish which causes neurotoxic shellfish poisoning (NSP) (Sayer, Qing et al. 2005). Besides consumption, humans are exposed to toxins in aerosol form from coastal spray. This phenomenon unique to *K. brevis* blooms may inflict “paresthesia, myalgia, vertigo, ataxia, pupil dilation, diarrhea, and headaches in exposed humans” (Sayer, Qing et al. 2005) (Pierce, Henry et al. 2005). It has also been shown that brevetoxin exposure can cause DNA damage to human lymphocytes (Sayer, Qing et al. 2005). Moreover, brevetoxins may persist in coastal waters for more than a month after the dissipation of a *K. brevis* bloom posing an even greater threat for exposure (Flewelling et al., 2005). Given red tide’s disastrous effects and with the frequency of

this destructive bloom increasing, it has become an ever more pressing matter to understand bloom dynamics. (Snyder, Guerrero et al. 2005).

To better understand bloom dynamics, it is necessary to study the environmental conditions that allow for bloom formation. *Karenia brevis* has a broad range of optimal growth conditions (Magana and Villareal 2006). The most important factors affecting growth are salinity and temperature with the optimal conditions being 25-40 ppt and 15-30 degrees Celsius respectively. Large optimal growth ranges gives *Karenia* an advantage as the earth's climate warms that may facilitate an increased frequency of *Karenia* blooms. Furthermore, pollutants dumped into aquatic systems can over-stimulate phytoplankton populations by providing them with an over- abundance of nutrients such as nitrate or phosphate. While these factors explain how environmental conditions are contributing to bloom formation, they do not explain how these phytoplankters are escaping the biotic pressures of their food web.

To understand how phytoplankton escape predation pressure, scientists are studying the relationships between phytoplankton and their consumers. Phytoplankton vary in shape, size, potential defenses, and nutritional value which can all “affect grazer feeding and fitness” (Prince, Lettieri et al. 2006). Therein, to understand bloom dynamics, we must understand how certain phytoplankton deter grazing. One way phytoplankton could deter grazing is by producing chemical defenses. Many recent studies have identified chemical defenses in macroscopic aquatic plants. For instance, Wilson and Fenical et al. showed that an organic extract from *Habenaria repens*, the freshwater orchid, deters feeding in crayfish (1999). More deterrent compounds, lipophilic and water-soluble, were isolated from *Saururus cernuus*, another freshwater

plant. These compounds also discouraged feeding in the crayfish, *Procambarus clarkii* (Kubaneck, Fenical et al. 2000). While the role of chemical defenses in macroscopic aquatic plants has been well studied, defenses in microscopic plants are not as well documented. Several strains of the phytoplankton, *Alexandrium sp.*, were found to be deterrent to the rotifer, *Brachionus plicatilis* (Wang, Yan et al. 2005).

Research has shown that *Karenia brevis* deters grazers (Kubaneck et al. in press). However, the deterrent properties are not due to brevetoxins. It seems that *Karenia brevis* defends itself by becoming a non-nutritious food source. For instance, when single diets of *Karenia brevis* were fed to the copepod, *Acartia tonsa*, there was decreased copepod egg production even though feeding rates increased (Prince, Lettieri et al. 2006). Moreover, when *A. tonsa* was fed other phytoplankton in addition to *Karenia brevis* the copepods seemed to be avoiding *K. brevis* consumption as if they knew not to eat it (Prince, Lettieri et al. 2006). Furthermore, a study by Giner et al. proposed that *Karenia brevis*, like many other aquatic organisms, has evolved unique types of sterols that may serve to render this plant mal-nutritious for its consumers (2003). Prince et al. supported this research when they found that nutritional inadequacy and not toxicity was responsible for copepod deterrence of *Karenia brevis* diets (2006). A recent study by Kubaneck et al. has furthered these studies by finding that *Karenia* cellular extracts are actually responsible for feeding deterrence in the rotifer, *B. plicatilis*. With this previous research in mind, it is our aim to isolate the cellular compound responsible for predator feeding deterrence in *Karenia brevis*.

Materials and Methods

Phytoplankton

Karenia brevis, originally from Sarasota Bay, Florida, USA, (CCMP strain 2228, cell volume $4.0 \times 10^3 \mu\text{m}^3$ calculated from dimensions $24.4 \times 24.4 \times 12.9 \mu\text{m}$ (Prince et al. 2006)) and *Rhodomonas lens*, originally from the Bahamas, (CCMP strain 739, cell volume $290 \mu\text{m}^3$ calculated from dimensions $12.4 \times 6.7 \times 6.7 \mu\text{m}$ (Prince et al. 2006)) were obtained from the Provasoli- Guillard National Center for Culture of Marine Phytoplankton (CCMP). Both strains were incubated at 22 °C Percival incubator with a 12 h light/ 12 h dark cycle. The incubator contained Philips F32T8/TL741 Universal/ Hi-vision fluorescent bulbs mounted vertically. Both strains were grown in autoclaved L1 media made with filtered natural seawater collected in Maine, USA (32-35 ppt). Culture growth was monitored using chlorophyll fluorescence measurements (Turner Designs TD-700 calibrated with chlorophyll A standard). Phytoplankton concentration was accessed using a Palmer-Maloney settling chamber and an Olympus IX-50 inverted microscope.

Rotifers

Brachionus plicatilis (strain RUS) was originally collected from the Azov Sea in Russia. Rotifers were left to hatch from resting eggs in 25 ppt filtered seawater for 48 hours in the 22 °C Percival incubator described above. Rotifers were placed in experimental wells of filtered Maine seawater (32-35 ppt) using a pipette.

Preparation of *K. brevis* cellular extracts

Before beginning our experiment, we tested cellular extract prepared previously by Kubanek et al. for activity using a bioassay as described below. The lost

deterrence of this cellular extract led us to prepare fresh extract from a *Karenia brevis* culture. When the *Karenia brevis* culture was in early stationary phase (22,000 cells/ml), an Amicon ultrafiltration unit was used to slowly filter 970 ml of culture through a 5 µm Millipore Isopore TMTP membrane until 10-20% of the starting volume remained as retentate as previously described (Kubanek et al., in press 2007). Once the cells had been rinsed with filtered natural seawater, the liquid was filtered through until about 100 ml remained. The concentrated cells were then lysed with 100 ml of deionized water, and organic compounds released by cell lysis were extracted for 17 h at 5 °C with a mixture of absorbent resins (equal proportions of Diaion HP-20 (Supelco), Amberlite XAD-7-HP (Acros Organics), Amberlite XAD-16 (Supelco), totaling about 150 ml of wet resin) (Prince et al., 2006). A Nitex nylon mesh with a pore size of 160 µm was used to collect the resin, which was then rinsed with deionized water, and the compounds were eluted from the resin using methanol and acetone (about 300 ml each). The solvents were removed by rotary evaporation yielding a cellular extract of *K. brevis*. The frozen crude extract was re-dissolved with methanol and then partitioned into 14 separate aliquots (each containing the extracted organic compounds from 60 ml of *K. brevis* culture, hereafter known as 60 ml equivalents of cellular extract).

Bioassay- Guided Fractionation

Fractionation: Several fractionation techniques were used to isolate and partially characterize the deterrent compound. Each technique outlined below resulted in at least two fractions that were each tested in a bioassay for deterrence. The first fractionation technique used was membrane dialysis which was performed using a Spectra/Por Membrane with a molecular weight cut-off of 1000 Daltons. 400 ml of water

was placed outside the membrane, while the extract dissolved in minimal amounts of water was placed inside the membrane. The membrane was connected to a stirring rod that moved continuously. The dialysis was performed over night in 5 degrees Celsius. Another technique, liquid partition paired ethyl acetate and water to separate the compound into two fractions based on polarity. 100 ml of each solvent was combined in a separatory funnel and then dried down by rotary evaporation. As the ethyl acetate fraction showed activity, we used this fraction in high performance liquid chromatography (HPLC) with a ZORBAX SB- C18 column and an eluent gradient of 50:50 to 100:0 methanol: water. We then used another ethyl acetate fraction and partitioned it by size using a LH-20 Sephadex column with a 400:100:40 eluent ratio of ethyl acetate: methanol: water. This column yielded 16 fractions, so the fractions were pooled into 6 fractions using TLC with 95:5 dichloromethane: methane as the solvent to detect similar properties.

Preparation of Yeast-Based Diet for Bioassay: To determine whether a fraction contained feeding deterrent compounds, rotifers were fed re-hydrated yeast particles (Culture Selco, INVE Inc.) treated with a particular fraction. The amount of a fraction added to the yeast corresponded to the amount of extract derived from a similar biomass of *K. brevis* cells as previously described (Kubanek et al., in press). About 60 ml equivalents of each fraction were dissolved in a small amount of methanol. Then about 2.3 mg of yeast was added, and the mixture was sonicated. Then, the solvent was evaporated using a Savant Speed-Vac concentrator. Seawater was then used to hydrate the extract-treated yeast. The yeast was added to the wells 2 hours prior to anything else to allow them time to settle and stick (Kubanek et al., in press). These hydrated yeast

particles were always paired with a known good food, *R. lens* (about 200,000 cells/ ml), in experimental wells. In addition to the fraction treated yeast, each bioassay contained three control treatments. One control contained *R. lens* and yeast that were treated with methanol but with no fractions. Another control held *R. lens* and yeast treated with all the fractions together (recombined). Rotifers were fed only once in each bioassay. Lastly, every bioassay contained a starvation control (n=6) that received no food and only seawater to account for the volume differences.

Bioassay: After each fractionation technique, each fraction was tested against the rotifer, *B. plicatilis*, for the presence of a deterrent compound by measuring rotifer feeding behavior and reproduction. After each diet was prepared, as described above, approximately 10 rotifers were added to each well. Each experiment was conducted in 24- well sterile polystyrene plates (Corning) with about 1.5 ml total volume per well. Each diet was replicated in at least 6 wells, and treatments and controls were interspersed within 24 well plates. Once the rotifers and an appropriate diet had been added to each well, several factors were examined including the total number of live rotifers, the total number of live rotifers with food in their guts, and the number of rotifers carrying eggs. These factors were monitored at various time intervals under an Olympus SZ61 dissecting light microscope at 10x magnification.

Statistics

Feeding activity was analyzed using one-way repeated measures ANOVA after arc-sin transformation of proportional data. Egg production was examined using a one-

way ANOVA then Tukey post-hoc test on arc-sin transformed proportional data. All statistical analyses used Systat and ($p \leq 0.05$) as the mark of significance.

Results

We obtained cellular extract prepared previously by Kubanek et al., and we tested this extract for activity. The results of our bioassay showed this cellular extract to have lost deterrence during storage (Figure 1). Despite a negative feeding trend within the cellular extract diet, there was no significant difference between this extract diet and the positive control, *R. lens* with untreated yeast ($p = 0.125$). Given that several months prior this extract was shown to be active (Kubanek et al. in press), we believe the deterrent compound(s) decomposed over time. So, a new extract was prepared and tested for activity (Figure 2). This cellular extract was deterrent, since rotifer feeding behavior was significantly suppressed when fed extract-coated yeast, relative to a positive control ($p \leq 0.001$).

Bioassay-guided fractionation has identified several characteristics about the deterrent compound. When we used membrane dialysis and liquid partition to identify broad characteristics of the deterrent compound(s), we found that each of the size-based fractions moderately deterred rotifer feeding when compared to the recombined fractions diet and the positive control diet (Figure 3). One-way repeated measures ANOVA followed by post-hoc analysis declared the relationship between these treatments to be significant ($p \leq 0.001$).

Liquid partition yielded two fractions that had completely different effects on rotifer feeding. Rotifers fed fractions containing compounds dissolved in water showed

no deterrent feeding activity (Figure 2). However, rotifers fed fractions containing compounds dissolved in ethyl acetate strongly avoided eating (Figure 2). As the deterrent compound(s) was present in the nonpolar solvent, the unknown compound itself is probably nonpolar. To determine how complex the fraction containing compounds dissolved in ethyl acetate was, TLC plates were performed and each yielded three spots. Because the ethyl acetate-soluble fraction was deterrent, we used ethyl acetate fractions for the remainder of our fractionation techniques. HPLC yielded eight fractions that were tested in two separate bioassays. None of the fractions significantly deterred feeding in rotifers ($p \leq 0.464$ for Graph A) ($p \leq 0.585$ for Graph B) (Figure 5). There was however a significant time effect for both bioassay groups ($p \leq 0.001$ for both Graphs), and an insignificant synergistic effect between time and treatment ($p \leq 0.833$ for Graph A), ($p \leq 0.334$ for Graph B). Our last fractionation attempt used a Sephadex LH-20 column which yielded 16 fractions. These fractions were then grouped into 6 fractions using TLC. The recombined fraction diet and the fraction 6 diet both showed deterrent activity (Figure 5). Both were significantly different from the positive control diet as well as the other diets ($p \leq 0.001$) ($p \leq 0.001$). Both of these treatments were unusual in that after the one hour time point the rotifers seemed to have died. .

We decided to also run an ethyl acetate fraction from liquid partition with the Sephadex LH-20 column bioassay to test for decomposition. This diet was not active and is very similar to the positive control diet (Figure 6). It seems that at this time, we have lost activity in our crude extract and will have to make more.

Discussion

Our experimentation has determined several characteristics about the unknown deterrent compound. We now have evidence that the compound is probably small (Fig. 3), nonpolar (Fig. 4), and unstable (Fig. 1, 5). Characterizing this compound increases our understanding of phytoplankton defenses against predators. This knowledge may help to explain how phytoplankton populations are escaping trophic pressures and exploding into harmful blooms. For instance, evidence has shown that phytoplankton toxins, rather than being toxic, may work by changing consumer behavior, reducing food intake, or reducing reproductive ability (Turner et al. 1998). In the presence of active fractions, our rotifers behaved differently than those in other treatments. These rotifers appeared bloated and spent much of their time resting on the bottom of the wells. These rotifers also ate significantly less food than other treatments which could be responsible for their observed reduction in reproductive out-put. Lower egg-production leads to smaller population growth. Smaller population growth means rotifers will not be able to replace themselves effectively in the next generation. The result is a population of rotifers that cannot keep up with the growing numbers of phytoplankton. If a predator cannot check phytoplankton growth, the phytoplankton population will be limited only by nutrient availability.

Bloom dynamics are extremely complicated. Evidence suggests that many different grazers may be present in bloom situations. These grazers may all be affected differently by the toxins produced by phytoplankton in these bloom situations (Turner et al. 1998). Given the wide variety of effects these phytoplankton toxins can have on their predators, choosing an appropriate model to study these interactions can be very difficult. Recently, there has been much discussion about the validity of using zooplankton to

model interactions between phytoplankton and their consumers. Turner proposes that mesozooplankton, like the rotifer, play an insignificant role in phytoplankton consumption, and their deterrence may therefore be inconsequential for explaining bloom formation (2006). However, Turner also argues that rotifers have the ability to become incredible phytoplankton consumers because they can reproduce by parthenogenesis when the proper conditions for growth are met. Furthermore, Arndt indicates that rotifers may already be significant feeders on smaller marine organisms (1993).

Since rotifers are common to estuarine and coastal waters (Turner 2006), they will only be exposed to blooms when the blooms have moved closer to shore. *K. brevis* blooms usually begin in deeper waters where rotifers are not abundant, but they can move closer to shore through currents and wind giving the rotifer adequate time to interact with this phytoplankton species (Tester and Steidinger 1997). Then, the rotifer's explosive growth capacity could drastically impact the bloom's duration. Furthermore, rotifers are excellent predator models because they are not selective feeders unlike other common phytoplankton grazers (Starkweather 1980). Their role as unselective feeders makes them an important tool for studying the fitness effects of certain phytoplankton diets because they will either eat uniformly or eat nothing at all. In general, we believe that rotifers serve as good models for grazer interactions with phytoplankton. There is a wide body of evidence to suggest that many "toxic" effects induced by phytoplankton to their consumers are usually species specific. But, even if the observed deterrence is specific to this species of rotifer, characterizing the deterrent compound still furthers knowledge on how phytoplankton use chemicals to defend themselves against predators.

Debates on the role of zooplankton predation in bloom formation have been closely tied to studies on the role of phytoplankton “toxins” in defense against predators. Recent research has down- played the role of phytoplankton toxins as a chemical defense against predators. For instance, there is an extremely wide variety of phytoplankton toxins, and most of these toxins are harmful to only certain grazers. The variety of effects these toxins have on zooplankton consumers leads many researchers to believe that these compounds may not have evolved as defenses against predation (Turner et al. 1998). Instead, they could have evolved for many other reasons including bioluminescence, nitrogen storage, pheromones, or chromosomal structural organization (Turner 2006). However, even if these toxins did not evolve originally for chemical defense and the effect is merely coincidental, its role in deterring predator feeding should not be ignored. Whether intentional or not, the negative effects induced by these chemicals are responsible for altering the dynamics of zooplankton populations. Even if zooplankton are not important controllers of bloom formation, the identification of potential chemical defenses shows that phytoplankton are applying selective pressures that are shaping the characteristics of these zooplankton populations. And, as zooplankton are vitally important to marine food webs, anything that may be potentially shaping the characteristics of these populations should be studied and understood.

Our unknown compound has been shown to have negative effects on rotifer fitness. When we fed *B. plicatilis* diets of *Karenia brevis* cellular extract, there was a significant decrease in reproduction when compared to other control diets (data not shown). This means that in subsequent generations the ability of rotifers to replace themselves will be limited. We could apply these results to bloom situations. For

instance, if rotifers cannot replace themselves, they will not be able to help control the growing population of phytoplankton. However, recent research by Turner has asserted that models using only one zooplankton predator is not accurate for describing a bloom situation. He argues that in bloom situation there would be many predators all with different reactions to this harmful chemical (2006). The inability of one zooplankton predator, like the rotifer, to feed would not make a substantial contribution to bloom formation. Furthermore, he asserts that the effects of one harmful phytoplankton species would be diluted by the presence of other phytoplankton species in a natural bloom situation. We assert however that studying individual predators especially when paired with more than one phytoplankton diet can still be beneficial. The findings will begin to paint a more accurate picture of what may be occurring during bloom formation. For instance, we now know that *Karenia brevis* reduces feeding activity in the presence of the rotifer *Brachionus plicatilis*. Prince et al. showed that *Karenia brevis* reduced reproductive out-put in the copepod, *A. tonsa* (2006). Our unknown compound may not be deterrent for every predator of *Karenia brevis*, but the predators that are affected could certainly make an impact on the dynamics in bloom formation. If more than one potential predator is incapacitated by this species, then the potential for phytoplankton to grow unchecked increases. As more data becomes available for predator reactions to *Karenia brevis* diets, we will begin to understand how all these factors contribute to bloom formation. Isolating this deterrent chemical is only a small piece in the explanation of bloom formation. We hope that further characterization will lead to more ideas that can then be applied to other predator models. Further predator models may

give insight into the significance of this deterrent compound in understanding the mechanisms of *Karenia brevis* bloom formation.

Figures

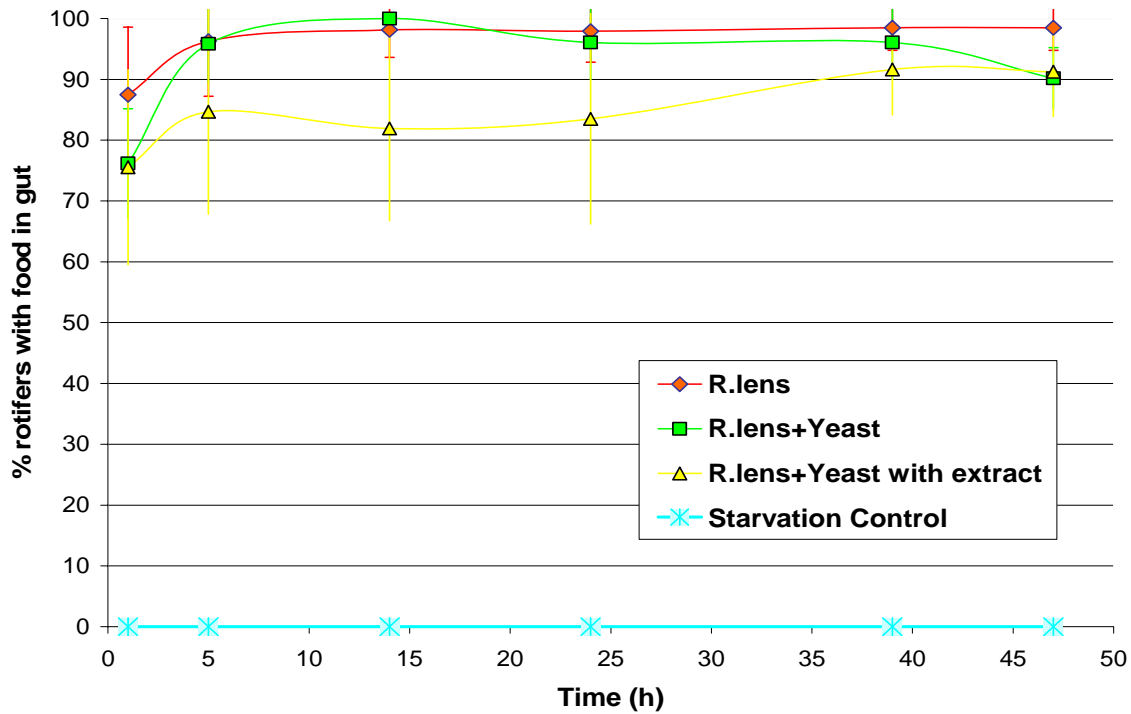


Figure 1. Feeding behavior of rotifers exposed to chemical defenses of *K. brevis* as part of a yeast-based diet (mean \pm SD; n=6). One-way repeated measures ANOVA showed a significant difference between treatments ($p = 0.025$), but no significant difference when just diets of *R. lens* and yeast were compared to diets of *R. lens* and yeast-coated extract ($p = 0.125$). Both comparisons showed a significant time effect and synergistic time with diet effect ($p \leq 0.001$) and ($p = 0.055$).

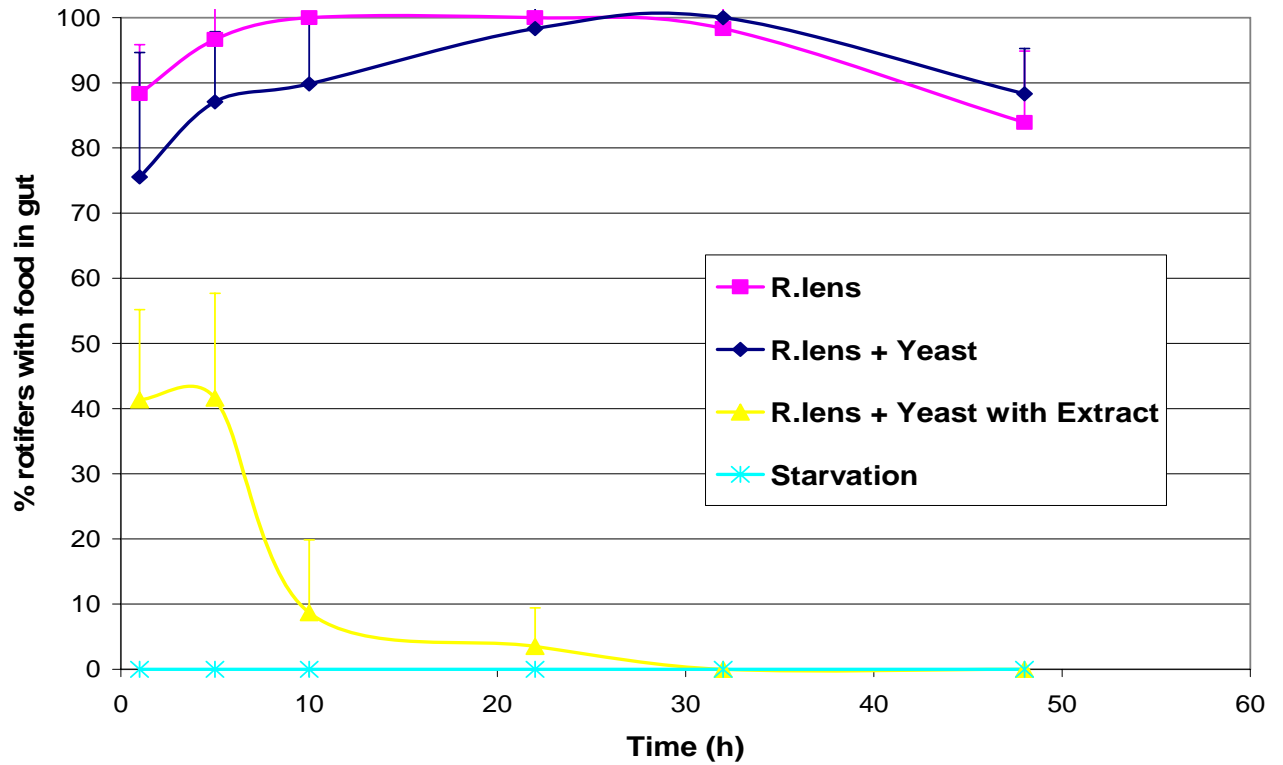


Figure 2. Feeding behavior of rotifers exposed to *K. brevis* extract coated onto yeast particles and offered with a diet of live *R. lens* (mean \pm SD, n=6). The diet of extract-treated yeast was significantly different from the control diets, *R. lens* alone and *R. lens* with yeast ($p \leq 0.001$). There was also a significant time effect ($p \leq 0.001$) and synergistic effect between time and diet ($p \leq 0.001$).

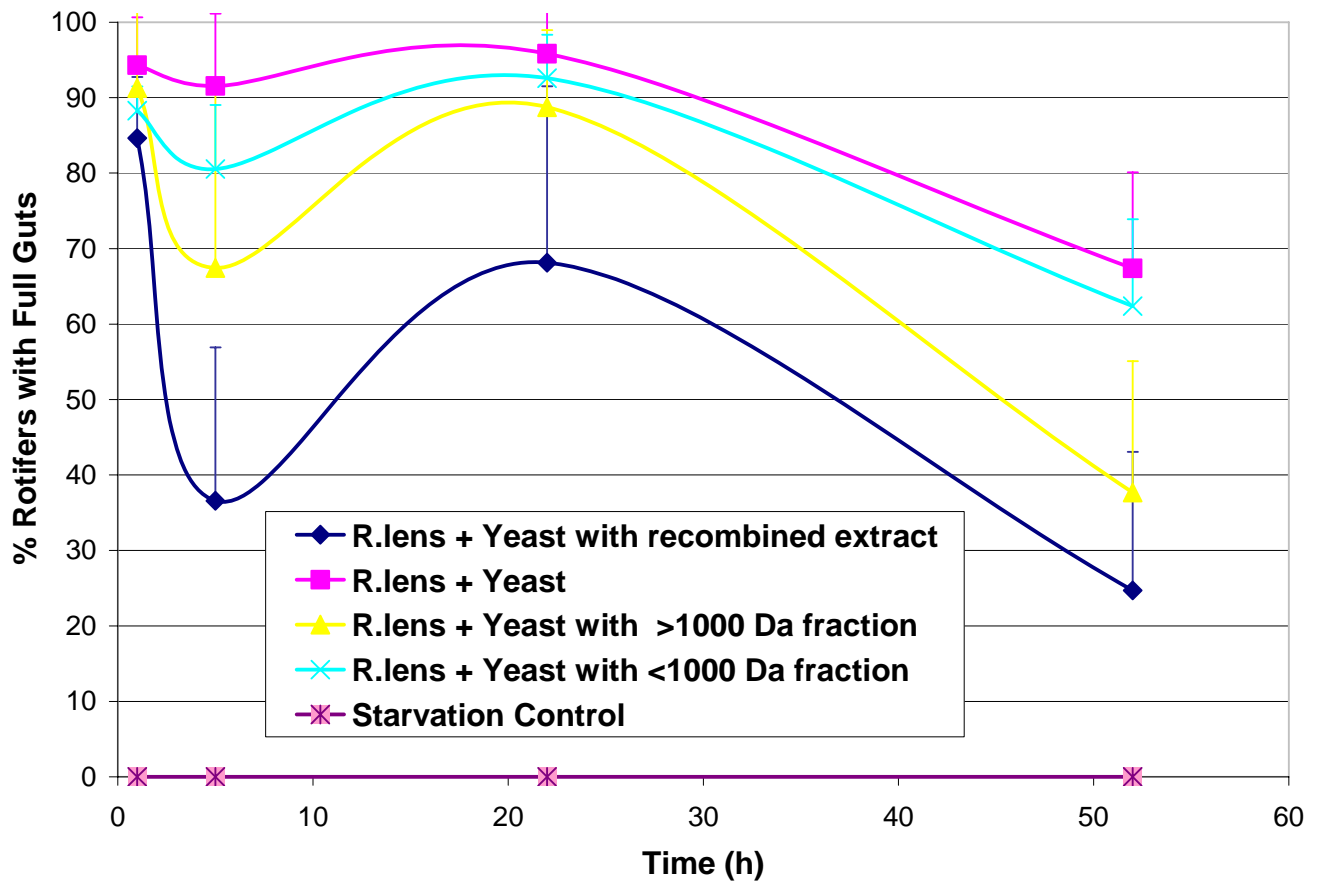


Figure 3. Feeding behavior of rotifers exposed membrane dialysis fractions coated onto yeast and paired with live *R.lens* (mean \pm SD, n=6). One-way repeated measures ANOVA detected a significant difference between treatments ($p \leq 0.001$), a time significance ($p \leq 0.001$), and a significant time*diet interaction ($p = 0.039$).

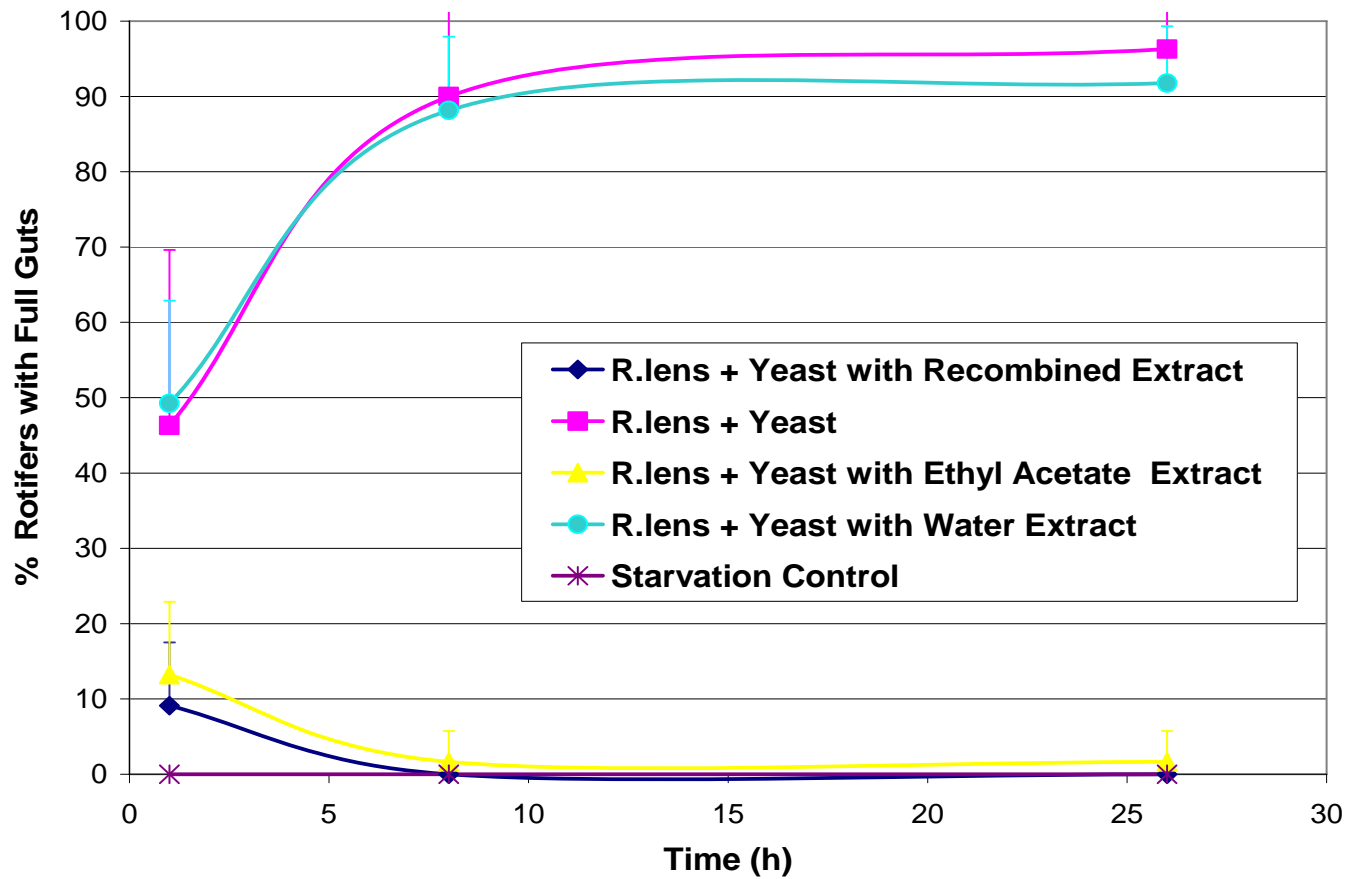
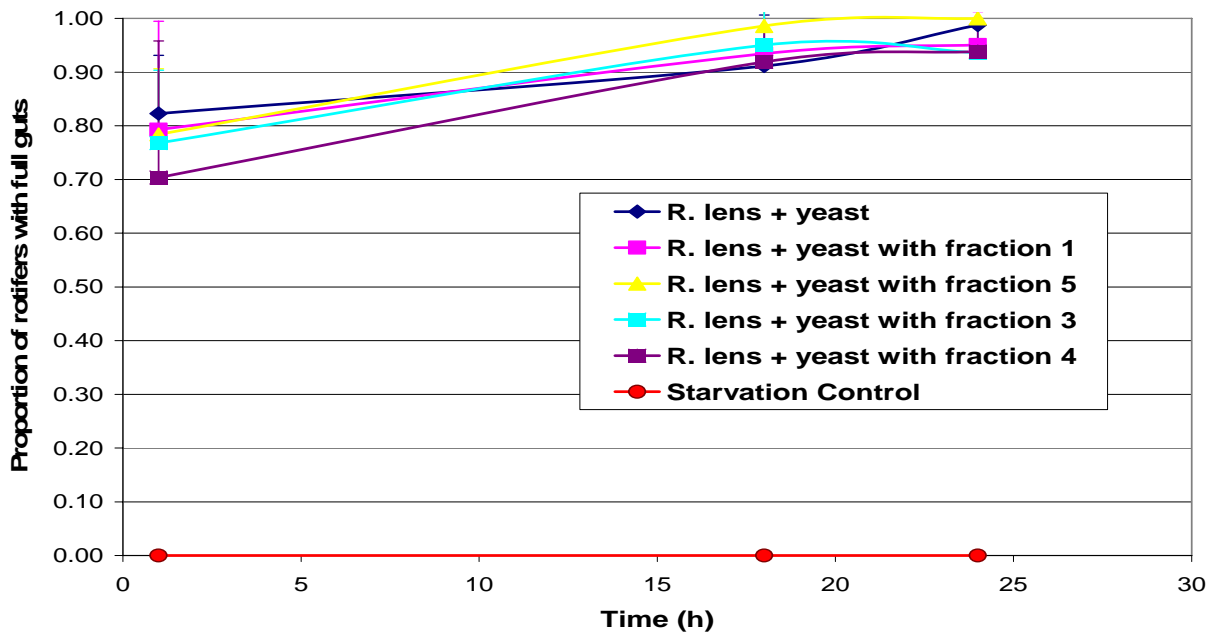


Figure 4. Rotifer feeding behavior measured in the presence of liquid partition fractions coated onto yeast particles along with a live *R.lens* diet (mean \pm SD, n=6). One-way repeated measures ANOVA identified a significant difference between treatments ($p \leq 0.001$), a time significance ($p \leq 0.001$), and a significant time*diet interaction ($p = 0.039$).

A.



B.

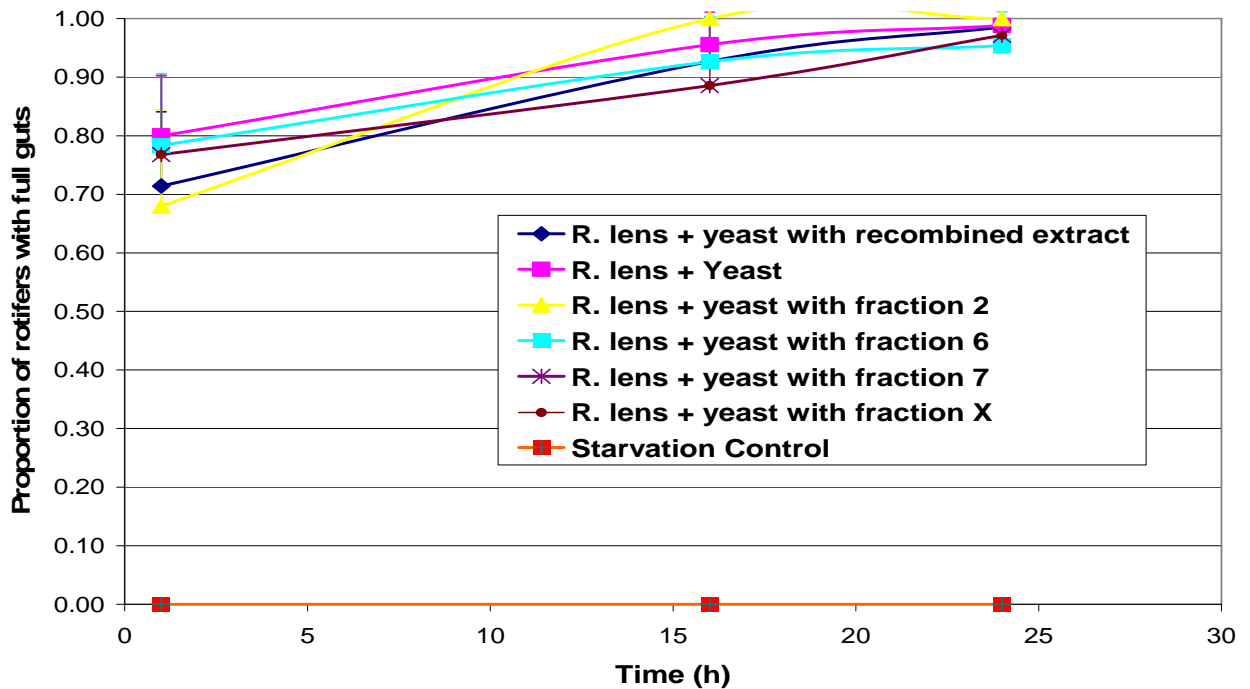
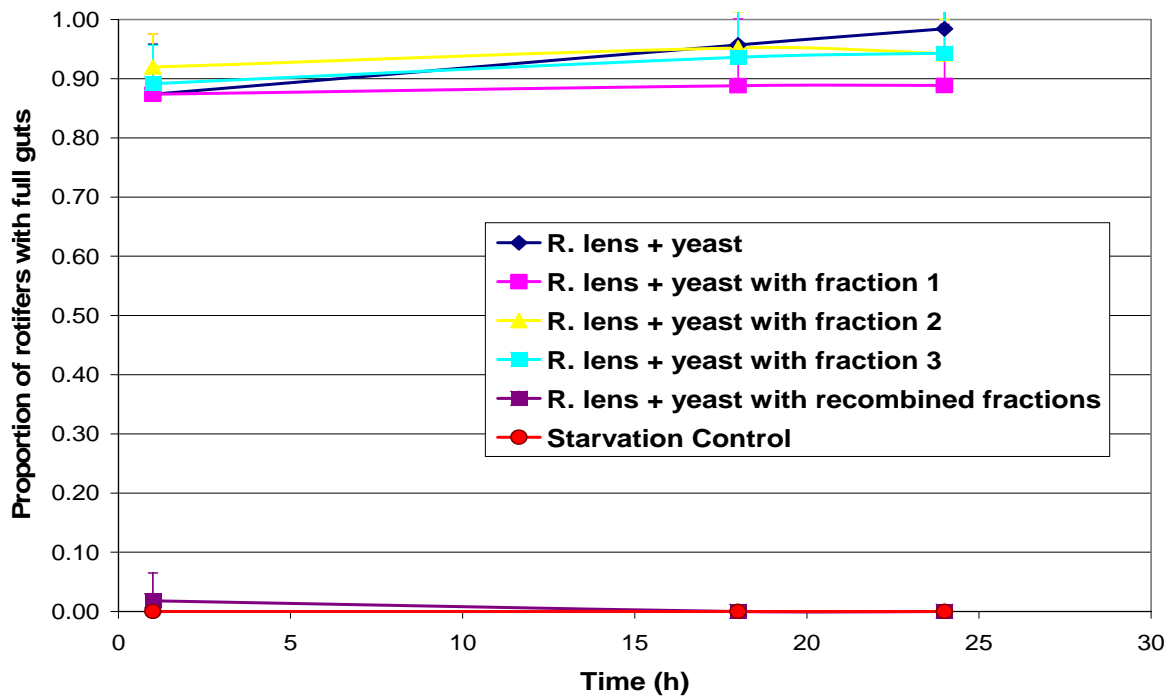


Figure 5. Feeding activity of rotifers exposed to diets of HPLC fractions coated onto yeast and live *R. lens* (mean \pm SD, n=6). Statistical analyses using one-way repeated measures ANOVA recognized an insignificant difference between treatments ($p = 0.464$) ($p = 0.585$), a significant difference between time points ($p \leq 0.001$) ($p \leq 0.001$), and an insignificant synergistic effect between time and treatments ($p = 0.833$) ($p = 0.334$) for Graphs A and B respectively.

A.



B.

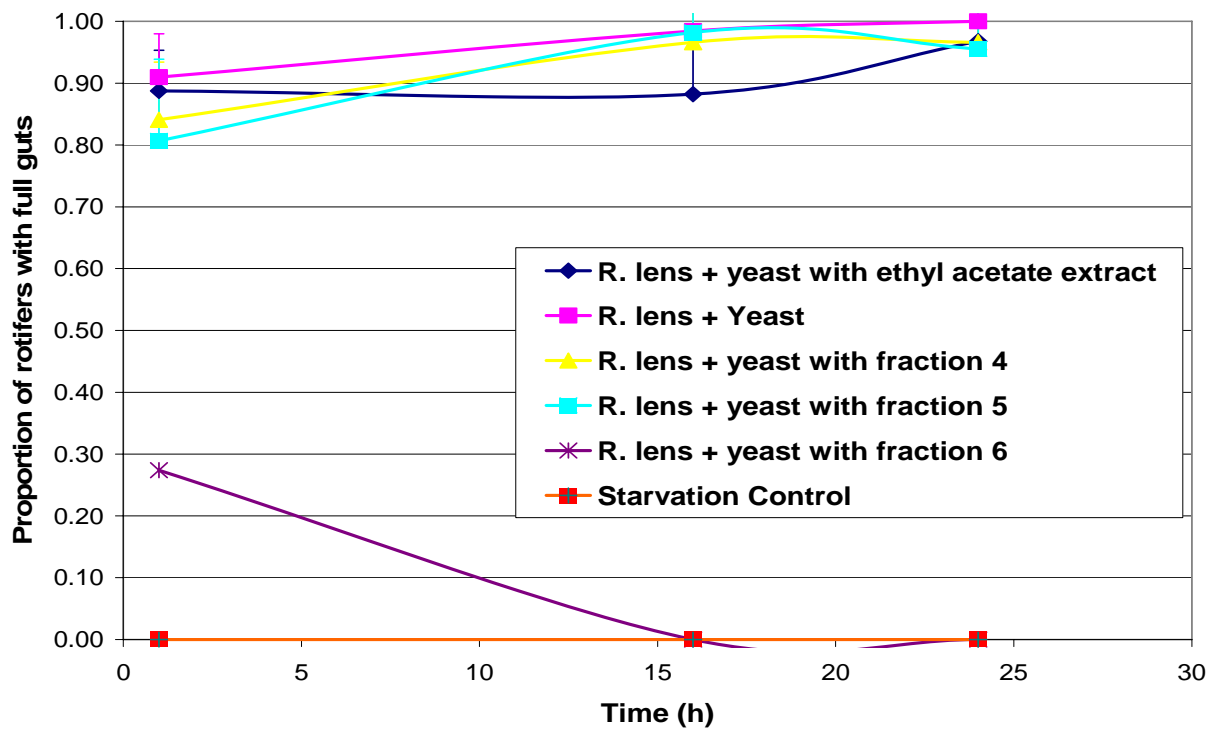


Figure 6. Rotifer feeding behavior when exposed to diets consisting of LH-20 column fractions coated onto yeast particles and live *R. lens* (mean \pm SD, n=6). An ethyl acetate fraction from liquid partition is also added as a treatment to this bioassay. Statistical analyses using one-way repeated measures ANOVA recognized a significant difference between treatments ($p \leq 0.001$) ($p \leq 0.001$), a significant difference between time points ($p \leq 0.006$) ($p \leq 0.001$), and a significant synergistic effect (for Graph B) between time and treatments ($p = 0.250$) ($p \leq 0.001$) for Graphs A and B respectively.

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